Redistribution of pulmonary EC-SOD after exposure to asbestos

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Submitted 4 May 2004; accepted in final form 28 July 2004

Tan, Roderick J., Cheryl L. Fattman, Simon C. Watkins, and Tim D. Oury. Redistribution of pulmonary EC-SOD after exposure to asbestos. J Appl Physiol 97: 2006–2013, 2004. First published August 6, 2004; doi:10.1152/japplphysiol.00480.2004.—Inhalation of asbestos fibers leads to interstitial lung disease (asbestosis) characterized by inflammation and fibrosis. The pathogenesis of asbestosis is not fully understood, but reactive oxygen species are thought to play a central role. Extracellular superoxide dismutase (EC-SOD) is an antioxidant enzyme that protects the lung in a bleomycin-induced pulmonary fibrosis model, but its role has not been studied in asbestos-mediated disease. EC-SOD is found in high levels in the extracellular matrix of lung alveoli because of its positively charged heparin-binding domain. Proteolytic removal of this domain results in clearance of EC-SOD from the matrix of tissues. We treated wild-type C57BL/6 mice with 0.1 mg of crocidolite asbestos by intratracheal instillation and euthanized them 24 h later. Compared with saline- or titanium dioxide-treated control mice, bronchoalveolar lavage fluid (BALF) from asbestos-treated mice contained significantly higher total protein levels and increased numbers of inflammatory cells, predominantly neutrophils, indicating acute lung injury in response to asbestos. Decreased EC-SOD protein and activity were found in the lungs of asbestos-treated mice, whereas more EC-SOD was found in the BALF of these mice. The EC-SOD in the BALF was predominantly in the proteolyzed form, which lacks the heparin-binding domain. This redistribution of EC-SOD correlated with development of fibrosis 14 days after asbestos exposure. These data suggest that asbestos injury leads to enhanced proteolysis and clearance of EC-SOD from lung parenchyma into the air spaces. The depletion of EC-SOD from the extracellular matrix may increase susceptibility of the lung to oxidative stress during asbestos-mediated lung injury.

Extracellular superoxide dismutase; acute lung injury; antioxidants; oxidative stress; asbestosis; proteolysis; pulmonary fibrosis

Asbestosis is a form of interstitial lung disease caused by the inhalation of asbestos fibers. Characterized by inflammation and pulmonary fibrosis, asbestosis is a debilitating disease that causes significant morbidity and mortality in patients and additionally leads to an increased risk of lung cancer (27). It is estimated that ~27 million workers in the United States were exposed to asbestos between 1940 and 1979 (31). Because the latency between asbestos exposure and disease development ranges from 20 to 40 yr, this disease will remain a significant health problem (22, 42).

The molecular events underlying asbestosis are largely unknown. However, current evidence supports a role for reactive oxygen species (ROS) (reviewed in Refs. 10, 24). ROS can oxidatively modify proteins, lipids, and nucleic acids, altering their normal functions (44). Asbestos fibers, particularly the highly fibrogenic amphibole types, possess a high iron content that can catalyze the production of hydroxyl radicals through the Haber-Weiss reaction (18). Schapira et al. (41) provided evidence for this hydroxyl radical formation in rat lungs after asbestos exposure. White blood cells are also a source of ROS (19), and inhalation of asbestos fibers leads to an intense inflammatory reaction in the lung (6). Previous studies have shown that asbestos directly increases ROS generation in both human neutrophils (38) and alveolar macrophages (17). A protective effect has been observed for manganese superoxide dismutase, catalase, and iron chelators such as deferoxamine in various models of asbestos-induced cellular damage (29, 30, 36). Collectively, these data suggest that an oxidant-antioxidant imbalance is a key event in the pathogenesis of asbestosis.

Extracellular superoxide dismutase (EC-SOD) is a 135-kDa antioxidant enzyme that scavenges the superoxide free radical and is expressed in especially high levels in mammalian lungs. EC-SOD exists primarily as a homotetramer (33), and each subunit contains a positively charged heparin-binding domain that confers affinity for the extracellular matrix (ECM) (reviewed in Ref. 12). This heparin-binding domain can be posttranslationally cleaved without affecting enzyme activity (40).

EC-SOD has been shown to be protective in several models of interstitial lung injury (5, 13). However, the role of EC-SOD in models of pneumoconiosis has not been examined. Here we report a redistribution of this antioxidant from the ECM of the lung into the alveolar lining fluid after intratracheal instillation of asbestos due to proteolysis of the heparin-binding domain. Thus proteolytic clearance of EC-SOD from the pulmonary interstitium after acute asbestos injury may contribute to the oxidant-antioxidant imbalances that could then lead to pulmonary fibrosis.

Materials and Methods

Materials. Crocidolite asbestos fibers (>10 μm in length) were obtained from the National Institutes of Environmental Health Sciences. Xanthine oxidase was from Boehringer Mannheim (Indianapolis, IN). Eosin Y, phloxine B, xanthine, equine partially acetylated cytochrome c, Ponceau S, chloramine T, methyl cellosolve, titanium dioxide, and anti-β-actin antibody were purchased from Sigma (St. Louis, MO). Mayer’s hematoxylin, 10% buffered formalin, and p-dimethylaminobenzaldehyde were from Fisher Scientific (Pittsburgh, PA). Clear Rite was obtained from Richard-Allan Scientific (Kalamazoo, MI). Nova Red staining kit and ABC immunohistochemistry

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compared with both saline and titanium dioxide controls at 24 h, ANOVA with Tukey’s fi

Increased lung injury and inflammation 24 h and 14 days after exposure to asbestos

Male C57BL/6 mice (Taconic, Germantown, NY), 8–10 wk old, were treated with 0.1 mg of asbestos, 0.1 mg titanium dioxide, or 0.9% saline vehicle by intratracheal instillation as previously described (1). Mice were euthanized 24 h or 14 days later, as indicated. Bronchoalveolar lavage fluid (BALF) was obtained by the intratracheal instillation and recovery of 0.8 ml 0.9% saline. Lungs were removed and flash frozen in liquid nitrogen and stored at −70°C until used for biochemical analyses as described below. Lungs from some mice were inflation fixed with 10% buffered formalin and paraffin embedded for histological analysis.

Bronchoalveolar lavage fluid. Total protein was determined by use of the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Total white blood cell counts were obtained with a Beckman Z1 Coulter particle counter (Beckman Coulter, Fullerton, CA). To obtain a differential count, BALF samples were adhered to glass slides with a cytospin, and numbers of macrophages, neutrophils, lymphocytes, and eosinophils were counted under a microscope.

EC-SOD isolation. EC-SOD was isolated and concentrated as previously described (11). Briefly, lungs were homogenized and sonicated in 50 mM potassium phosphate with 0.3 M potassium bromide, pH 7.4. After an aliquot was removed for Western blotting, the remaining homogenate was passed over a concanavalin A Sepharose column that binds EC-SOD but not other superoxide dismutases. After elution from the column, the separated EC-SOD was concentrated and assayed for superoxide dismutase activity.

EC-SOD activity. EC-SOD activity from lung homogenates was determined as described previously (9). In this assay, EC-SOD activity was measured by inhibition of the reduction of partially acetylated cytochrome c by superoxide generated by the reaction of xanthine oxidase on xanthine at pH 10.

Western blot analysis. Analysis was performed as previously described (34). Briefly, 10 µg protein from BALF or lung homogenates were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). EC-SOD was detected and assayed for superoxide dismutase activity.

Western blot analysis. EC-SOD was isolated and concentrated as previously described (11). Briefly, lungs were homogenized and sonicated in 50 mM potassium phosphate with 0.3 M potassium bromide, pH 7.4. After an aliquot was removed for Western blotting, the remaining homogenate was passed over a concanavalin A Sepharose column that binds EC-SOD but not other superoxide dismutases. After elution from the column, the separated EC-SOD was concentrated and assayed for superoxide dismutase activity.

RESULTS

Intratracheal instillation of asbestos causes lung inflammation. To assess the acute response of the lung to asbestos, protein and inflammatory cell accumulation in the BALF were determined. Compared with saline-treated controls, asbestos-treated mice possessed significantly greater BALF total protein (Table 1). In addition, these mice exhibited greater numbers of cells in their BALF. After application of BALF to glass slides with a cytospin, it was revealed that almost all of these cells were leukocytes. The majority of leukocytes were identified as neutrophils. To ensure that the changes observed were not due to a nonspecific response to particles in the lung, the experiments were repeated using titanium dioxide as an inert particulate control (21). Although the neutrophil counts in titanium dioxide-treated mice were elevated with respect to saline-treated mice, this increase was not significant (P > 0.05). The

Table 1. Increased lung injury and inflammation 24 h and 14 days after exposure to asbestos

<table>
<thead>
<tr>
<th></th>
<th>BALF Protein, mg</th>
<th>Total WBC Cells (×10⁶)</th>
<th>Macrophages Cells (×10⁶)</th>
<th>Lymphocytes Cells (×10⁶)</th>
<th>PMN Cells (×10⁶)</th>
</tr>
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<tbody>
<tr>
<td>Saline, 24 h</td>
<td>0.107±0.004</td>
<td>282.4±23.8</td>
<td>276.2±23.6</td>
<td>3.2±0.8</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td>Titanium dioxide, 24 h</td>
<td>0.145±0.002</td>
<td>298.8±73.4</td>
<td>176.5±33.0</td>
<td>3.0±0.4</td>
<td>117.7±59.5</td>
</tr>
<tr>
<td>Asbestos, 24 h</td>
<td>0.592±0.044*</td>
<td>642.6±53.1*</td>
<td>62.5±10.5*</td>
<td>5.6±1.1</td>
<td>573.0±53.4*</td>
</tr>
<tr>
<td>Titanium dioxide, 14 days</td>
<td>0.122±0.003</td>
<td>332.9±37.6</td>
<td></td>
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</tr>
<tr>
<td>Asbestos, 14 days</td>
<td>0.330±0.0381</td>
<td>508.7±48.7</td>
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</tr>
</tbody>
</table>

Values are means ± SE. Mice were intratracheally instilled with either saline vehicle or titanium dioxide (0.1 mg) as controls or with crocidolite asbestos (0.1 mg) and were euthanized 24 h or 14 days after this instillation as indicated. Data are combined from multiple experiments. PMN, neutrophils. *P < 0.05 compared with both saline and titanium dioxide controls at 24 h, ANOVA with Tukey’s posttest. Saline values were not significantly different from titanium dioxide values at 24 h. T²P < 0.05 compared with titanium dioxide at 14 days. Student’s t-test.

J Appl Physiol • VOL 97 • NOVEMBER 2004 • www.jap.org
number of neutrophils in asbestos-treated mice, however, was significantly greater compared with these titanium dioxide-treated mice. Histological analysis of lung tissue from asbestos-treated mice revealed asbestos fibers along with interstitial thickening and inflammation (Fig. 1). Collectively, these data indicate that intratracheal instillation of asbestos leads to significant pulmonary damage as well as inflammation dominated by neutrophils 24 h after the injury.

**EC-SOD is depleted from the lung parenchyma after asbestos exposure.** EC-SOD activity in lung tissue was significantly diminished in asbestos-treated mice compared with controls (Fig. 2). When EC-SOD protein abundance was compared by Western blotting, lung homogenates from mice treated with asbestos had significantly decreased EC-SOD compared with both saline- and titanium dioxide-treated controls (Fig. 3). Coincident with this depletion from the lung was a significant increase in BALF levels of EC-SOD in asbestos-treated mice compared with saline-treated controls (Fig. 4A). This increase was also present when BALF levels of EC-SOD were normalized to total protein loading as determined by Ponceau S staining of Western blots (data not shown). In the BALF, there was an increased ratio of cut to uncut EC-SOD in the asbestos mice compared with controls, indicating increased proteolysis of EC-SOD (Fig. 4B). Similar results were obtained when titanium dioxide was used as a control instead of saline alone (not illustrated). Because proteolysis of EC-SOD removes the heparin-binding domain that confers affinity for the ECM, proteolysis may therefore lead to a redistribution of EC-SOD from lung parenchyma into the air spaces.

Immunohistochemistry for EC-SOD confirmed our Western blot and EC-SOD activity results. Quantification of EC-SOD labeling revealed strong staining intensity for EC-SOD in the alveolar septa of saline-treated mice, matching data our laboratory has previously published (11, 34). In contrast, the septa of asbestos-treated mice had significantly decreased staining intensity, indicating a loss of EC-SOD from these areas (Fig. 5).

**Redistribution of EC-SOD in the lung is associated with development of pulmonary fibrosis.** To determine whether redistribution of EC-SOD correlates with the development of pulmonary fibrosis in response to asbestos (asbestosis), we examined mice 14 days after a single intratracheal instillation of either titanium dioxide or asbestos (0.1 mg). BALF protein and white blood cell counts were slightly attenuated in asbestos-treated mice at 14 days compared with asbestos-treated mice at 24 h (Table 1). However, levels of both of these parameters were significantly increased over 14-day titanium dioxide-treated control mice. EC-SOD Western blots also revealed greater amounts of EC-SOD in the BALF of asbestos-treated mice compared with controls (Fig. 6), indicating that EC-SOD continued to accumulate in the BALF 14 days after exposure to asbestos.

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**Fig. 1.** Asbestos induces pulmonary inflammation. Hematoxylin and eosin staining was performed on lung sections from mice treated with saline (A) or with asbestos (B and C). Asbestos fibers are shown with an arrow. Boxed area (in C) is shown at greater magnification (in D) to show fibers in detail. Bar equals 25 μm.

**Fig. 2.** Asbestos exposure reduces extracellular superoxide dismutase (EC-SOD) activity in lungs. Lungs (n = 9 for each group) were obtained and homogenized 24 h after asbestos treatment. After EC-SOD isolation and concentration, activity was assessed via the inhibition of cytochrome c reduction by superoxide at pH 10. Error bars denote SE. *P < 0.05, Student's t-test.
Histological analysis was performed on these mice to determine whether fibrosis developed 14 days after exposure to asbestos. As shown in Fig. 7, asbestos-treated mice possessed increased pulmonary fibrosis compared with titanium dioxide controls. Hydroxyproline levels were also measured to quantify fibrosis and revealed significantly greater collagen deposition in asbestos-treated mice over controls (Fig. 8).

Fig. 3. Asbestos decreases total EC-SOD protein in the lung. Western blot analysis was performed using 5 μg of total lung protein [n = 4 for saline and titanium dioxide (TiO₂), n = 5 for asbestos]. Densitometry was normalized to β-actin as a loading control. Error bars denote SE. *P < 0.05, ANOVA followed by Tukey’s posttest.

Fig. 4. Asbestos exposure results in increased levels of EC-SOD in bronchoalveolar lavage fluid (BALF). Note also the increased ratio of cut to uncut EC-SOD after asbestos treatment (bottom band on blot and bottom graph), indicating that asbestos leads to increased proteolysis of EC-SOD’s heparin-binding domain. Western blots were performed with 10 μg BALF protein. Similar results were obtained by using TiO₂ instead of saline. Error bars denote SE. *P < 0.05, Student’s t-test, n = 5 for saline, n = 7 for asbestos.
DISCUSSION

Asbestos-associated lung diseases are significant health problems. The ~30 million tons of asbestos that have been used in the United States since 1900 have led to both occupational and nonoccupational exposures to these toxic fibers. Although the use of asbestos has been declining in the United States, the long latency between exposure and development of disease ensures that a number of new cases will continue to arise (reviewed in Ref. 23).

An oxidant-antioxidant imbalance is theorized to underlie the pathogenesis of asbestos-mediated lung disease. Asbestos exposure in vitro and in vivo leads to ROS formation detectable through markers of oxidative stress (16, 26). Asbestos also upregulates antioxidants such as manganese superoxide dismutase and glutathione peroxidase, signifying a compensatory response to oxidative stress (20).

Evidence from our laboratory and others implicates EC-SOD in fibrotic and acute lung injuries. In a bleomycin model of pulmonary fibrosis, we have reported a decrease in EC-SOD protein levels in the lung ECM. This is associated with an increase in the proteolyzed form of EC-SOD lacking the heparin-binding domain in both the lung and BALF (9). Depletion of EC-SOD from lung ECM was also found in a hyperoxic model of acute lung injury (34). These results suggest that increased proteolysis of EC-SOD leads to its removal from the lung parenchyma, perhaps leading to increased oxidative stress in the ECM. In support of a protective role for EC-SOD against lung injury, transgenic overexpression of EC-SOD (4) or treatment with superoxide dismutase mimetics (35) protects against bleomycin-induced pulmonary fibrosis, whereas EC-SOD knockouts are more susceptible (8). In this report, we test the hypothesis that asbestos exposure leads to a loss of EC-SOD from the lung parenchyma that can lead to increased oxidative stress in the lungs.

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Fig. 5. Asbestos leads to decreased EC-SOD in alveolar septa. Immunoperoxidase labeling for EC-SOD (dark staining) was performed on lungs from mice treated with saline (n = 6) or with asbestos (n = 5). Note the decreased labeling intensity for EC-SOD in mice treated with asbestos compared with saline-treated controls. Labeling intensity is expressed as percent EC-SOD staining per threshold area. Bar equals 50 μm. Error bars denote SE. *P < 0.05, Student’s t-test.

Fig. 6. EC-SOD levels in the BALF continue to be elevated 14 days after exposure to asbestos. Mice were treated with 0.1 mg TiO₂ or asbestos and euthanized 14 days later. Western blots were performed on BALF, loading 3 μg total protein for each sample. These data are representative of 2 individual experiments. Error bars denote SE. *P < 0.05, Student’s t-test, n = 4 for each group.
In our mouse model, we observed that asbestos initiates an early inflammatory, primarily neutrophilic, response with associated protein accumulation in the air spaces 24 h after exposure. This confirms results from Dorger et al. (6), who also found a neutrophilic influx at 24 h in rats after intratracheal instillation of asbestos. Lung histology revealed an influx of leukocytes as well as some interstitial thickening in areas infiltrated by asbestos fibers.

Coincident with this acute inflammation, we observed a depletion of EC-SOD from lung parenchyma by activity assays, immunohistochemistry, and Western blot analysis. In an attempt to localize EC-SOD, we examined the air spaces through BALF recovery. In the BALF, we found an accumulation of proteolyzed EC-SOD. This finding suggests that full-length EC-SOD is being proteolyzed and released from the ECM of the lung in response to asbestos-induced lung injury. The EC-SOD then accumulates in the air spaces, resulting in high amounts of proteolyzed EC-SOD in the BALF. This redistribution of EC-SOD may leave the lung parenchyma vulnerable to oxidative stress and promote the development of fibrotic injury.

The exact protease cleaving the heparin-binding domain of EC-SOD in our model is currently unknown, but a number of candidates are suggested by other investigations as well as the data presented here. Recent studies have implicated a role for furin in intracellular proteolysis of EC-SOD before secretion into the ECM (3, 7). Neutrophils also possess numerous proteases that may be released during the pulmonary inflammation accompanying asbestos exposure. In addition, asbestos fibers lead to extensive cytotoxicity (25), particularly in epithelial cells (2) and macrophages (15). These dying cells may release proteases into the ECM. Finally, asbestos exposure leads to increased vascular permeability and edema (6), which may introduce proteolytic enzymes from the circulation into the lung. Future studies will examine these possibilities in greater detail.

Although we have observed similar results in other inflammatory models of lung injury, it remains to be determined whether EC-SOD depletion from the lung contributes to inflammation or is a by-product of increased inflammation and release of proteolytic factors. We hypothesize that loss of EC-SOD can be proinflammatory. Collagen is one potential target of oxidative stress in asbestos lung injury and is present in high amounts in the ECM. Collagen fragments are known chemoattractants and activators of neutrophils (28). It has been shown that latent collagenases can be activated by ROS (14, 39) and that collagen itself can be directly fragmented by oxidative stress (43). EC-SOD has been shown to associate with type I collagen (32) and can prevent its oxidative fragmentation in vitro (37) and in vivo (8). Depletion of EC-SOD from the lung parenchyma could lead to increased collagen fragmentation and increased neutrophil chemotaxis and activation. It has already been shown that EC-SOD can inhibit...
pulmonary inflammation in response to hyperoxia (13) and bleomycin (8). In further support of this hypothesis, we find that neutrophil migration into the lung coincides with loss of EC-SOD from the lung in asbestos-treated mice (Table 1; Figs. 2, 3, 4A, 5). Neutrophils themselves can also produce large quantities of ROS (which can lead to more collagen fragmentation) and proteases (which can cleave collagen and can also potentially cleave the heparin-binding domain of EC-SOD, leading to its release from the lung parenchyma).

In summary, we report that a redistribution of pulmonary EC-SOD occurs 24 h after intratracheal instillation of asbestos. EC-SOD appears to be proteolytically removed from lung parenchyma with subsequent accumulation in the air spaces. The loss of this antioxidant from lung parenchyma may increase vulnerability of the lung parenchyma to oxidative stress. This change in localization of EC-SOD may contribute to the oxidant-antioxidant imbalance that contributes to asbestos-induced injury and may be a key early event in the progression to asbestosis and other asbestos-mediated diseases.

ACKNOWLEDGMENTS

We thank Lana Hanford, Toni Termin, Sean Alber, and Jason Callio for excellent technical assistance. We also thank Andrew Gioh for providing the asbestos used in these studies.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant R01-HL-063700 and an American Heart Association Established Investigator Award.

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